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We have isolated of a panel of peptides that bind selectively to angiogenic tumor vasculature. This was accomplished by using *in vivo* screening of peptide libraries in nude mice carrying human tumor xenografts. We have used two of the peptides that showed the highest specificity to target doxorubicin, a commonly used anthracyclin drug, into tumors. The peptides increase the potency of the drug while reducing its toxicity. Dramatic differences in mortality between tumor bearing-mice that received the drug conjugate relative to tumor-bearing mice treated with the free drug or with control conjugate were observed. These results indicate that tumor vasculature expresses a number of potential receptors for tumor targeting and that it may be feasible to develop targeted compounds with improved therapeutic profiles for cancer treatment.

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FOREWORD

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INTRODUCTION

Chemotherapy is the basis of the systemic treatment of disseminated tumors. However, a limitation of chemotherapeutic agents is their narrow therapeutic index. Anti-cancer agents are restricted by their nonselective effect on normal tissues. A more specific delivery of cytotoxics into tumors may overcome these limitations. Although this idea has been a long standing goal of medicine , there are only a few situations in which targeted drug delivery is possible. In contrast to the extensive existing work that explores tumor antigens, our approach to tumor targeting has been to search for small peptides that can directly home into tumors *in vivo*. Rather than targeting the tumor cells themselves, we target the endothelial lining of small blood vessels of tumors. The vasculature within tumors is distinct, presumably because it constantly undergoes neovascularization, forming new blood vessels required to allow the growth of the tumor. The distinct properties of the angiogenic neovasculature within tumors are reflected in the presence of specific markers in its endothelial cells and pericytes. It is those markers we are targeting. If the tumor vasculature is targeted, the killing of all target cells may not be required since partial denudation of the endothelium is likely to lead to the formation of an occlusive thrombus halting the blood flow through the entirety of the affected tumor vessel. The ability to detect tissue-specific endothelial molecules is based on the previous success of our laboratory in developing a method for *in vivo* selection of peptides from phage display peptide libraries. In a phage display peptide library, random peptides in as many as 10^9 permutations are expressed on the surface of phage by fusion of the peptide to one of the phage surface proteins. Phage capable of homing into certain organs or tumors are selected *in vivo* from such a peptide library following an intravenous injection. The ability of individual phage to target a tissue can also be analyzed by this method. Using the *in vivo* selection, it was possible to identify peptides that selectively target normal organs and tumors. The vasculature within solid tumors such as breast cancer carries specific targetable markers, presumably because of ongoing angiogenesis. We have isolated a panel of tumor targeting peptides by using the *in vivo* phage screening method and have selected a peptide for further development based on its high tumor homing selectivity. The results below suggest that when a cytotoxic drug is coupled to a targeting peptide, the resulting compound is more effective and causes fewer untoward effects than when the parental drug is administered alone in breast cancer. We determined the method-of-choice for preparation of the cytotoxic drug-tumor homing peptide compounds. We then treated cohorts of tumor-bearing mice on a dose escalation scheme to determine the efficacy of the drug-peptide compounds, perform *in vivo* distribution studies with radiolabeled compounds, and obtain preliminary toxicity in tumor-bearing mice.

BODY OF THE PROPOSAL

1. Description of the training

Wadih Arap, M.D., Ph.D. (Principal Investigator) has trained in internal medicine at the University of São Paulo and in the Medical Oncology and Hematology Fellowship Program at Memorial Sloan-Kettering Cancer Center (Program Director: Dr. George J. Bosl), and as a postdoctoral fellow in their Pharmacology Program (Chairperson: Dr. Joseph R. Bertino). Subsequently, he decided to pursue formal training in molecular oncology and earned his Ph.D. in Cancer Biology from Stanford University with the hope of developing improved cancer treatments. He worked in the genetic progression of malignant human tumors at the Ludwig Institute for Cancer Research-San Diego, under the guidance of Dr. Webster K. Cavenee. Dr. Arap has joined Dr. Erkki Ruoslahti's laboratory as a postdoctoral fellow, specifically to develop the targeted chemotherapy of malignant tumors. Within that group, Dr. Arap has been promoted to Senior Research Associate and then to Staff Scientist at The Burnham Institute. During his postdoctoral stay at the Burnham Institute, Dr. Arap developed the theoretical framework and laboratory skills to study extracellular matrix formation, cell adhesion molecules in tumors, angiogenesis, and vascular targeting of organs and tumors. This experience in an environment rich in extracellular techniques complemented his knowledge in intracellular work afforded by his Ph.D. training. Dr. Arap is committed to this novel tumor vasculature targeting approach; his dual background in cancer biology and medical oncology is highly advantageous to the project and it will allow the development of translation of the project into clinical applications solid tumors such as breast cancer. This project--as outlined in the Statement of Work--will continue in the laboratory of Dr. Erkki Ruoslahti at the Burnham Institute, under his outstanding guidance.

2. Results

Tumor-homing peptides: We have used nude mice bearing tumor xenografts derived from several human carcinoma, sarcoma, and melanoma cell lines to screen phage libraries for tumor targeting peptides. By screening the MDA-MB-435 tumors derived from human breast carcinomas, we have assembled a panel of motifs derived from various tumor screens. For a peptide to be considered as a candidate, it must be selected from the target tissues consistently over several rounds of selection, and its motif must appear frequently among other sequences analyzed. Subsequently, in order for a peptide to be considered a *bona fide* tumor targeting motif, three criteria must be fulfilled: enrichment in the tumor relative control organs (as measured by tetracycline resistant colony counting), inhibition of phage homing to the tumor by the cognate peptide, and immunohistochemical evidence of accumulation in the tumor vasculature. The experiments to isolate homing motifs were performed by quantitating the phage in tissue extracts after a short circulation time. This experimental time frame was chosen to avoid having

the specifically bound phage be taken up by the cells and inactivated. Perfusion through the heart of tumor-bearing mice decreased the phage counts in most tissues. As phage inactivation would not be critical in immunostaining experiments, we obtained immunostaining of tumors and control organs at various time points from 3 minutes to 24 hours using an anti-M13 antibody to detect the tumor homing phage. Strong phage staining in tumor vasculature was seen in short term experiments in which the CDCRGDCFC-phage was allowed to circulate for only 3-5 minutes, followed by perfusion. These staining was within the tumors, but not in normal endothelia. At 24 hours after intravenous phage injection, there was almost no phage left in the circulation and perfusion is not needed. Tumors contained substantial immunostaining of the RGD-4C phage. More recently, we have analyzed phage displaying a double-cyclic NGR-containing insert sequence (CNGRCVSGCAGRC peptide) in a similar manner as described above for RGD-4C phage. This yielded the highest tumor staining to control organ background ratio among all of our tumor homing peptides. We hypothesized that the active site in the peptide CNGRCVSGCAGRC was likely to be the CNGRC structure, because the amino acid sequence NGR (Asn-Gly-Arg) is almost exactly the amino acid sequence RGD (Arg-Gly-Asp) in reverse. The tripeptide NGR has also been previously shown by our group to have a weak affinity for some RGD-binding integrins. We tested this hypothesis by using two additional phage that displayed peptides with NGR motifs, but had no other similarity to the CNGRCVSGCAGRC-phage: a NGRAHA-phage and a CVLNGRMEC-phage. Both of these phage also targeted tumor vasculature. Phage displaying the double-cyclic CNGRCVSGCAGRC-peptide and the linear NGRAHA-peptide showed strong staining of the MDA-MB-435 derived tumors and no staining in brain and lung of the same mice. Several other control organs were also studied, and gave very low or no immunostaining, confirming the specificity of the NGR motif for tumor (but not normal) vessels. The immunostaining of the GSL targeting sequences has very different features, suggesting a different family of receptors. It will not be considered further here, but it will be the subject of other studies in our laboratory. On the basis of the above data, we synthesized the CNGRC peptide, found it to be highly active in inhibiting the homing of all the three NGR-containing phage to tumor vasculature. The distribution of the individual tumor targeting phage and various control phage were studied after intravenous injection into tumor-bearing nude mice. After intravenous administration and perfusion with DMEM, the phage was rescued from the tissues after 3-5 minutes, and the number of each tumor homing phage and various control phage (unselected mixtures or individual clones) per gram of tissue (or per ml, for whole-blood) was quantitated. The targeting ability of the CNGRCVSGCAGRC-phage yielded preferential homing to MDA-MB-435-derived breast carcinomas, relative to the brain. Brain and kidney were selected as a control organs because they have a low background retention of phage. The cognate cyclic peptide CNGRC inhibited the tumor homing of the CNGRCVSGCAGRC-phage, showing that the homing of this phage is dependent on the CNGRC sequence, not on some other feature of the peptide it displays or some coincidental property of the phage. The selectivity of individual targeting phage towards tumors is estimated based on multiple parameters. In

brief, (i) the number of phage recovered from tumor, brain and kidney; (ii) the background found in tumors, brain and kidney for unselected phage mixtures (which is higher for brain and kidney than for tumors); and (iii) the background of tumor targeting phage in the control organs. Our group reported that CDCRGDCFC-phage homes to proliferating blood vessels of solid tumors to levels of 40 to 80-fold greater than unselected control phage, as calculated from the equation [tumor targeting-phage/unselected phage (in tumors) x tumor targeting-phage/unselected phage (in control organs)], using results from a number of experiments. Most of the experiments reported for the CDCRGDCFC-phage were performed without DMEM perfusion through the heart. Instead, the tumor bearing-mice were snap frozen in liquid nitrogen shortly after the intravenous administration of the phage. Currently, we are using perfusion to increase the stringency of the phage selection and testing. Measured in this manner, the tumor/control organ ratio for the CNGRCVSGCAGRC-phage is about 9 and for the CDCRGDCFC-phage is about 3.5. Taking into account these ratios and the other factors discussed above, the overall tumor selectivity of the CNGRCVSGCAGRC-phage is estimated to be about 2 to 3-fold that of the CDCRGDCFC-phage. The tumor homing of the CNGRCVSGCAGRC-phage is inhibited by the CNGRC peptide, demonstrating the specificity of the phage binding. Unselected control phage mixtures (without multiple rounds of *in vivo* selection) show no appreciable enrichment to any particular tumor or organ and were used as a negative control. Other human and murine tumor types can be similarly targeted by the CDCRGDCFC-phage and the CNGRCVSGCAGRC-phage. In each case, the targeting was inhibited by the cognate peptide and the peptides had no measurable effect on the distribution of the unselected phage library mix. Moreover, incubation of the phage with peptides does not have an effect on phage viability or infectivity. To determine whether the interactive site responsible for the tumor homing of the NGR and the RGD phage were the same, cross-inhibition experiments with CNGRC and CDCRGDCFC peptides were performed. Cross inhibition was seen with the CDCRGDCFC peptide which, at very high doses, was able to inhibit the tumor homing of NGR-containing phage. The reciprocal inhibition, even at equivalent high doses of CNGRC peptide, was not observed. These results indicate that CNGRC and CDCRGDCFC peptides each target independent receptors or at least different binding sites in the same receptor. That leaves open the cloning and characterization of an as yet unidentified endothelial cell surface receptor(s) for the NGR ligands. Preliminary observations suggest that an excellent target receptor candidate for the NGR-ligands is CD13, an aminopeptidase. Aminopeptidases are proteases and several lines of evidence indicate that they may be important for angiogenesis. A class of cell surface-bound aminopeptidases is expressed in endothelial cells and upregulated in tumor vasculature. An obvious initial concern was related to species-specificity. For this tumor vasculature targeting system to be clinically useful, it is important that human endothelial cells can also be targeted by the isolated tumor homing peptides. We tested the ability of the various tumor-homing phage and peptides to bind to human vasculature by studying phage binding to histological tissue sections. The phage were incubated with unfixed paraffin-embedded slide sections

of human tumors and other tissues from cancer patients. Phage binding detected with a polyclonal antibody against the M13 phage. The results clearly indicate that our NGR and RGD-4C tumor targeting peptides bind to human tumor vasculature, but not normal blood vessels. Moreover, this technique will allow us to select candidate tumor types for targeted chemotherapy subsequent studies.

Drug targeting: As a proof-of-concept in a breast cancer-bearing mouse model, we have used the anthracyclin drug doxorubicin to demonstrate the feasibility of drug targeting. Several reasons have lead us to this choice. First, doxorubicin is still the most widely used cytotoxic agent, with the broadest spectrum of anti-tumor effects. Second, doxorubicin is the most effective single drug against human breast cancer, the basic animal model used in this proposal. Third, analytical methodology has been established based on the unique fluorophor characteristics of doxorubicin. Fourth, doxorubicin has been linked to several carriers such as monoclonal antibodies, hormone analogs, and dextran. Fifth, experiments on the chick chorioallantoic membrane (CAM) have demonstrated doxorubicin has anti-angiogenic activity. Based on the hypothesis that the destruction of solid tumors is mediated at least in part by local loss of vasculature, this anti-angiogenic activity of doxorubicin may be an advantage in vascular tumor targeting. We coupled two tumor-targeting peptides, CDCRGDCFC peptide (RGD-4C) and CNGRC to doxorubicin to determine if the peptides could improve the efficacy and/or toxicity profile of the drug. An unrelated cyclic peptide (GACVFSIAHECGA) was used to prepare a control conjugate. Extensive data are available for the RGD-4C conjugates. They show that the toxicity of doxorubicin is greatly reduced by the conjugation while the anti-tumor activity improves. The improvement was sufficient to ensure 100% survival of the conjugate group at a time when the controls had died of the tumor. More limited results are available for the CNGRC peptide (3 independent experiments), but the results indicate that the improvement in tumor response to chemotherapy and survival is as impressive as with the RGD-4C peptide. A significantly better survival was seen in the animals treated with the CNGRC-doxorubicin conjugate. We used a simple carbodiimide conjugation of the drug to the peptide. Also, we have administered very low doses of doxorubicin-equivalent, 5 μg/dose. The doxorubicin dose in the literature for tumor bearing-nude mice ranges between 50-200 μg/dose. Therefore, it is expected that optimal compound preparation and dose escalation of the conjugates will give even better results, perhaps leading to cures. We have selected the tripeptide NGR in its minimal cyclic form (CNGRC) as the primary targeting moiety in our lead drug-peptide compound. We have also selected the double-cyclic RGD peptide (CDCRGDCFC, RGD-4C) for the drug targeting studies because the immunostaining of tumors with the double-cyclic CDCRGDCFC-phage showed impressive results as well. Moreover, the cell surface receptor for the latter peptide is known (αv integrins in angiogenic tumor vessels). However, (i) the double-cyclic structure of the CDCRGDCFC peptide and the proper arrangement of its two disulfide bridges remains to be fully characterized, and (ii) CDCRGDCFC is a complex peptide, its synthesis in a homogeneous form has been plagued by a

difficult standardization, and low yields. In contrast, the single carboxyl group, single disulfide bridge structure of CNGRC is much simpler to synthesize and couple to a drug. Consequently, CNGRC will give better yields and be easier to standardize. Finally, our preliminary results using a doxorubicin-CNGRC conjugate show that the CNGRC peptide is at least as active (and perhaps more active) in enhancing doxorubicin activity than it is the double-cyclic CDCRGDCFC peptide. Therefore, CNGRC would seem to be a better choice for further development into a pharmaceutical than the RGD-4C peptide. However, we plan to develop both CNGRC and RGD-4C compounds in parallel. It is possible that certain human cancers will be more targetable by the RGD-4C peptide than by the CNGRC peptide, depending on the specific characteristics of their tumor vasculature. In addition, combination therapy with NGR-targeted and RGD-targeted compounds may have synergistic anti-tumor effects. Indeed, this is a likely possibility since the CNGRC and CDCRGDCFC peptides target different receptors. We hope that the specificity conferred by the tumor targeting peptides will lead to a reduction of the deleterious side-effects caused by chemotherapeutic agents such as doxorubicin. Moreover, the targeting of normal endothelial cells in the tumor vessels will circumvent the problems of primary or acquired drug resistance by the tumor cells.

KEY RESEARCH ACCOMPLISHMENTS

- Selecting a xenograft model from a panel of tumorigenic breast cancer cell lines
- Comparison of tumor-homing peptides in breast cancer-bearing mice
- Phage cross-inhibition experiments with the cognate peptides
- Conjugation of tumor-homing peptides (RGD-4C and NGR) to doxorubicin
- HPLC and NMR analysis of the conjugates
- Determination of sensitivity in vitro; comparison to controls
- Efficacy and toxicity in vivo (MDA-MB-435 model)
- Histological analysis (H&E) of treated tumors

REPORTABLE OUTCOMES

Manuscript

- **Arap W**, Pasqualini R, and Ruoslahti. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science*, 279:378-380, 1998 (Editorial concerning the publication: Barinaga M. Peptide-guided cancer drugs show promise in mice. *Science* 279:323-324, 1998)

Presentations

8/98 International Union Against Cancer, Rio de Janeiro, Brazil
9/98 CaP CURE Annual Meeting, Lake Tahoe, NV
11/98 Loma-Linda University, Riverside, CA
12/98 Memorial Sloan-Kettering Cancer Center, New York, NY
12/98 University of Texas M.D. Anderson Cancer Center, Houston, TX
1/99 International Agency for Research on Cancer, Lyon, France
1/99 University of Texas Southwestern, Dallas, TX
2/99 Free University Hospital, Amsterdam, The Netherlands
2/99 University of Lübeck, Lübeck, Germany
3/99 Louisiana State University Medical Center, Shreveport, LA
3/99 Scripps Research Institute, La Jolla, CA
7/99 Gordon Conference on Chemotherapy of Cancer, Colby Sawyer , NH
8/99 Department of Pathology, University of Vienna, Vienna, Austria

Employment/Research Opportunity

As of October 1999, Dr. Arap will start return to academic medicine at the University of Texas M. D. Anderson Cancer Center were he has been recruited as an Associate Professor of Medicine; he has also been given an independent research laboratory.

Cancer Treatment by Targeted Drug Delivery to Tumor Vasculature in a Mouse Model

Wadih Arap,* Renata Pasqualini,* Erkki Ruoslahti†

In vivo selection of phage display libraries was used to isolate peptides that home specifically to tumor blood vessels. When coupled to the anticancer drug doxorubicin, two of these peptides—one containing an α_v integrin–binding Arg-Gly-Asp motif and the other an Asn-Gly-Arg motif—enhanced the efficacy of the drug against human breast cancer xenografts in nude mice and also reduced its toxicity. These results indicate that it may be possible to develop targeted chemotherapy strategies that are based on selective expression of receptors in tumor vasculature.

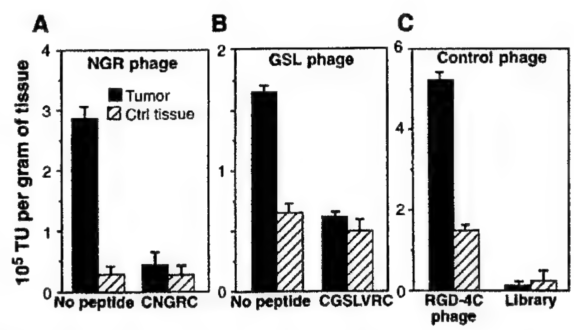
Endothelial cells in the angiogenic vessels within solid tumors express several proteins that are absent or barely detectable in established blood vessels (*1*), including α_v integrins (*2*) and receptors for certain angiogenic growth factors (*3*). We have applied in vivo selection of phage peptide libraries to identify peptides that home selectively to the vasculature of specific organs (*4*, *5*). The results of our studies imply that many tissues have vascular "addresses." To determine whether in vivo selection could be used to target tumor blood vessels, we injected phage peptide libraries into the circulation of nude mice bearing human breast carcinoma xenografts.

Recovery of phage from the tumors led to the identification of three main peptide motifs that targeted the phage into the tumors (*6*). One motif contained the sequence Arg-Gly-Asp (RGD) (*7*, *8*), embedded in a peptide structure that we have shown to bind selectively to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (*9*). Phage carrying this motif, CDCRGDCFC (termed RGD-4C), homes to several tumor types (including carcinoma, sarcoma, and melanoma) in a highly selective manner, and homing is specifically inhibited by the cognate peptide (*10*).

A second peptide motif that accumulated in tumors was derived from a library with the general structure $CX_3CX_3CX_3C$ (X = variable residue, C = cysteine) (*6*). This peptide, CNGRCVSGCAGRC, contained the sequence Asn-Gly-Arg (NGR), which has been identified as a cell adhesion motif (*11*). We tested two other peptides that contain the NGR motif but are otherwise different from CNGRCVSGCAGRC: a linear peptide, NGRAHA (*11*), and a cyclic peptide, CVLNGRMEC. Tumor homing for all three peptides was independent of the tumor type and species; the phage homed to a human breast carcinoma (Fig. 1A), a human Kaposi's sarcoma, and a mouse melanoma (*12*). We synthesized the minimal cyclic NGR peptide from the CNGRCVSGCAGRC phage and found that this peptide (CNGRC), when coinjected with the phage, inhibited the accumulation of the CNGRCVSGCAGRC phage (Fig. 1A) and of the two other NGR-displaying phages in breast carcinoma xenografts (*12*).

The third motif—Gly-Ser-Leu (GSL) and its permutations—was frequently recovered from screenings using breast carcinoma (*6*), Kaposi's sarcoma, and malignant melanoma, and homing of the phage was inhibited by the cognate peptide (Fig. 1B). This motif was not studied further here.

The RGD-4C phage homes selectively to breast cancer xenografts (Fig. 1C). This homing can be inhibited by the free RGD-4C peptide (*10*), but not by the CNGRC peptide, even when this peptide was used in amounts 10 times those that inhibited the homing of the NGR phage (Fig. 1D). Tumor homing of the NGR phage was also partially inhibited by the RGD-4C peptide (Fig. 1E), but this peptide was only 10 to 20% as potent as CNGRC. An unrelated cyclic peptide, GACVFSIAHECGA, had no effect on the tumor-homing ability of either phage (*12*). Thus, our in vivo screenings yielded two peptide motifs, RGD-4C and NGR, both of which had previously been reported



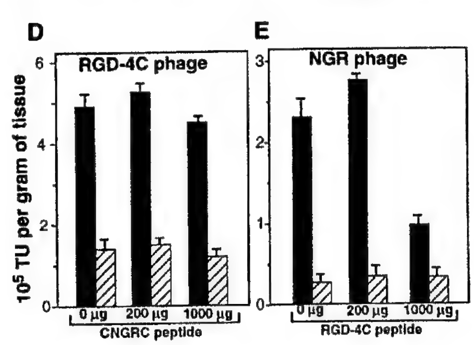


Fig. 1. Recovery of phage displaying tumor-homing peptides from breast carcinoma xenografts. Phage [10^9 transducing units (TU)] was injected into the tail vein of mice bearing size-matched MDA-MB-435–derived tumors (~1 cm^3) and recovered after perfusion. Mean values for phage recovered from the tumor or control tissue (brain) and the SEM from triplicate platings are shown. (**A**) Recovery of CNGRCVSGCAGRC phage from tumor (solid bars) and brain (striped bars), and inhibition of the tumor homing by the soluble peptide CNGRC. (**B**) Recovery of CGSLVRC phage and inhibition of tumor homing by the soluble peptide CGSLVRC. (**C**) Recovery of RGD-4C phage (positive control) and unselected phage library mix (negative control). (**D**) Increasing amounts of the CNGRC soluble peptide were injected with the RGD-4C phage. (**E**) Increasing amounts of the RGD-4C soluble peptide were injected with the NGR phage. Inhibition of the CNGRCVSGCAGRC phage homing by the CNGRC peptide is shown in (A); inhibition of the RGD-4C phage by the RGD-4C peptide has been reported (*10*).

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to bind to integrins (*9, 11*). The affinity of NGR for integrins is about three orders of magnitude less than that of RGD peptides (*7, 11*). Nevertheless, the homing ratio (tumor/control organ) of the phage displaying the NGR motif was three times that of the RGD-4C phage (*12*). This discrepancy in activities, and the cross-inhibition results described above, strongly suggest that the NGR and RGD-4C peptides bind to different receptors in the tumors.

We next studied phage homing to tumors by immunostaining (Fig. 2). In one set of experiments (*13*), phage was allowed to circulate for 3 to 5 min, followed by perfusion (*10*) and immediate tissue recovery. In the second set, tissues were analyzed 24 hours after phage injection, when there is almost no phage left in the circulation (*10*). Strong phage staining in tumor vasculature, but not in normal endothelia, was seen in the short-term experiments with CNGRCVSGCAGRC phage in MDA-MB-435 cell–derived human breast carcinoma xenografts (Fig. 2A) and SLK cell–derived human Kaposi's sarcoma xenografts (Fig. 2B). The two other NGR phages, NGRAHA and CVLNGRMEC, also showed strong tumor staining (*12*), whereas a control phage showed no staining (Fig. 2, E and F). At 24 hours, the staining pattern indicated that the NGR phage had spread outside the blood vessels and into the tumors (Fig. 2, C and D). This spreading may be attributable to increased permeability of tumor blood vessels (*14*) or uptake of the phage by angiogenic endothelial cells (*15*) and subsequent transfer to tumor tissue.

The CNGRCVSGCAGRC phage showed the greatest tumor selectivity among all the peptides analyzed. Several control organs showed very low or no immunostaining, confirming the specificity of the NGR motif for tumor vessels; heart (Fig. 2G) and mammary gland (Fig. 2H) are shown (*16*). Spleen and liver, which are part of the reticuloendothelial system (RES), contained phage; uptake by the RES is a general property of the phage particle and is independent of the peptide it displays (*10, 17*). These immunostaining results with the NGR phage are similar to observations made with the RGD-4C phage (*10*).

To determine whether the tumor-homing peptides RGD-4C and CNGRC could be used to improve the therapeutic index of cancer chemotherapeutics, we coupled them to doxorubicin (dox) (*18*). Dox is one of the most frequently used anticancer drugs and one of a few chemotherapeutic agents known to have antiangiogenic activity (*19*). The dox-peptide conjugates were used to treat mice bearing tumors derived from human MDA-MB-435 breast carcinoma cells.

The commonly used dose of dox in nude mice with human tumor xenografts is 50 to 200 μg/week (*20*). Because we expected the dox conjugates to be more effective than the free drug, we initially used the conjugates at a dose of dox-equivalent of only 5 μg/week (*13, 21*). Tumor-bearing mice treated with RGD-4C conjugate outlived the control mice, all of which died from widespread disease (Log-Rank test, $P < 0.0001$; Wilcoxon test, $P = 0.0007$) (Fig. 3A). In a dose-escalation experiment, tumor-bearing mice were treated with the dox-RGD-4C conjugate at 30 μg of dox-equivalent every 21 days for 84 days and were then observed, without further treatment, for an extended period of time. All of these mice outlived the dox-treated mice by more than 6 months, suggesting that both primary tumor growth and metastasis were inhibited by the conjugate. Many of the tumors in the mice that received the dox-RGD-4C conjugate (30 μg of dox-equivalent every 21 days) showed marked skin ulcer-

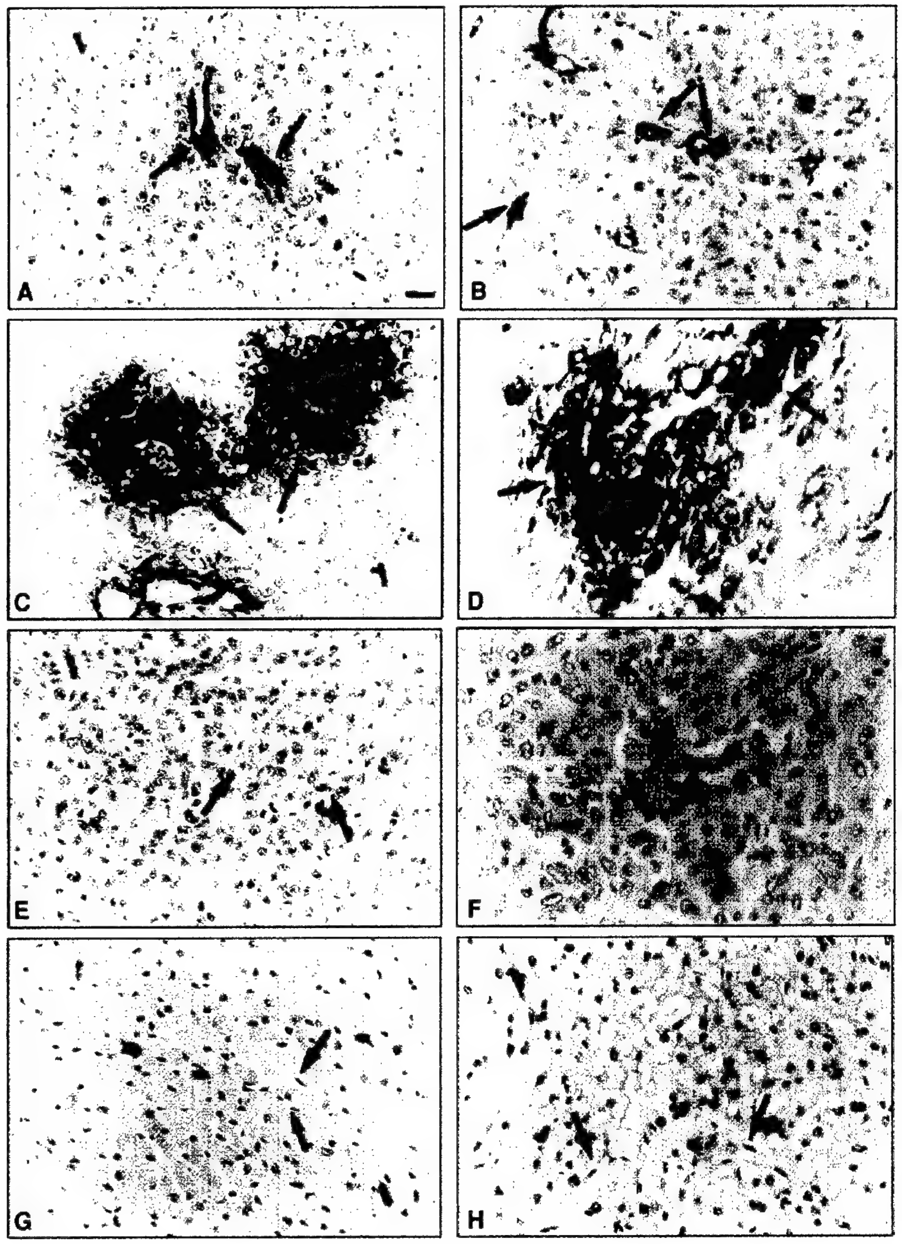


Fig. 2. Immunohistochemical staining of phage after intravenous injection into tumor-bearing mice. Phage displaying the peptide CNGRCVSGCAGRC (**A** to **D**, **G**, and **H**) or control phage with no insert (**E** and **F**) were injected intravenously into mice bearing MDA-MB-435–derived breast carcinoma (A, C, and E) and SLK-derived Kaposi's sarcoma (B, D, and F) xenografts. Phage was allowed to circulate for 4 min (A, B, E, and F) or for 24 hours (C, D, G, and H). Tumors and control organs were removed, fixed in Bouin solution, and embedded in paraffin for preparation of tissue sections. An antibody to M-13 phage (Pharmacia) was used for the staining. Heart (G) and mammary gland (H) are shown as control organs (*16*). Arrows point to blood vessels. Scale bar in (A), 5 μm.

ation and tumor necrosis, whereas these signs were not observed in any of the control groups. At necropsy, the mice treated with the dox-RGD-4C conjugate had significantly smaller tumors (t test, $P = 0.02$), less spreading to regional lymph nodes ($P < 0.0001$), and fewer pulmonary metastases ($P < 0.0001$) than did the mice treated with free dox (Fig. 3, B to D). Similar results were obtained in five independent experiments. Histopathological analysis revealed pronounced destruction of the tumor architecture and widespread cell death in the tumors of mice treated with the dox-RGD-4C conjugate; tumors treated with free dox at this dose were only minimally affected. In contrast, the dox-RGD-4C conjugate was less toxic to the liver and heart than was free dox (Fig. 3E). In some experiments, dox together with unconjugated soluble peptide was used as a control; the drug-peptide combination was no more effective than free dox (*12*).

To assess toxicity, we used 200 μg of dox-equivalent in mice with large (~5 cm^3), size-matched tumors (*13*, *21*). Mice treated with the dox-RGD-4C conjugate survived more than a week, whereas all of the dox-treated mice died within 48 hours of drug administration (Fig. 3F). Accumulation of dox-RGD-4C within the large tumors thus appeared to have sequestered the conjugated drug, thereby reducing its toxicity to other tissues.

Less extensive data with the CNGRC peptide conjugate indicated an efficacy similar to that of the RGD-4C conjugate. In all experiments, tumors treated with the dox-CNGRC conjugate were one-fourth to one-fifth as large as tumors treated in the control groups (Fig. 4A). A marked reduction in metastasis and a prolongation of long-term survival were also seen (Log-Rank test, $P = 0.0064$; Wilcoxon test, $P = 0.0343$) (Fig. 4B). Two of the six dox-CNGRC–treated animals were still alive more than 11 weeks after the last of the control mice died. The dox-CNGRC conjugate was also less toxic than the free drug (Fig. 4C). CNGRC peptide alone failed to reproduce the effect of the conjugate, even in doses up to 150 μg/week. Unconjugated CNGRC-dox mixture

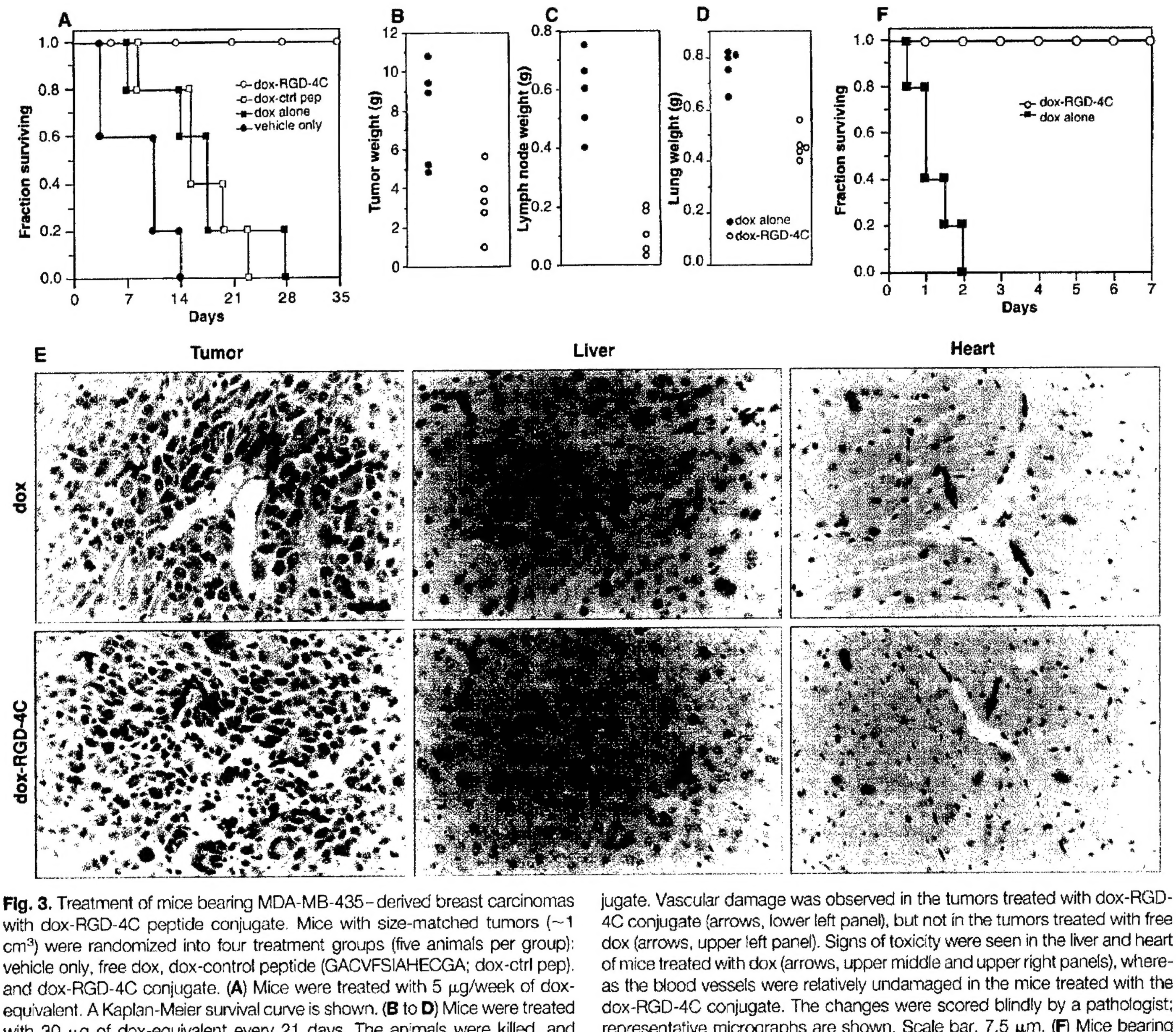
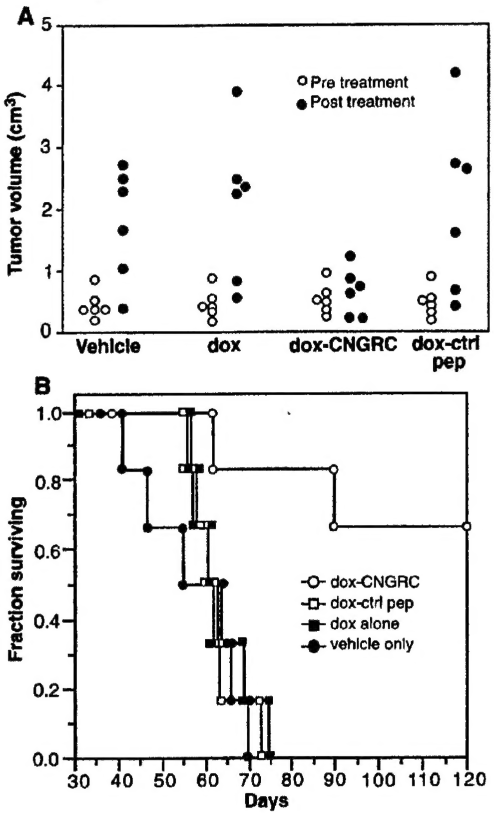


Fig. 3. Treatment of mice bearing MDA-MB-435–derived breast carcinomas with dox-RGD-4C peptide conjugate. Mice with size-matched tumors (~1 cm^3) were randomized into four treatment groups (five animals per group): vehicle only, free dox, dox-control peptide (GACVFSIAHECGA; dox-ctrl pep), and dox-RGD-4C conjugate. **(A)** Mice were treated with 5 μg/week of dox-equivalent. A Kaplan-Meier survival curve is shown. **(B** to **D)** Mice were treated with 30 μg of dox-equivalent every 21 days. The animals were killed, and tumors (B), axillary lymph nodes (C), and lungs (D) were weighed after three treatments. **(E)** Histopathological analysis (hematoxylin and eosin stain) of MDA-MB-435 tumors, liver, and heart treated with dox or dox-RGD-4C conjugate. Vascular damage was observed in the tumors treated with dox-RGD-4C conjugate (arrows, lower left panel), but not in the tumors treated with free dox (arrows, upper left panel). Signs of toxicity were seen in the liver and heart of mice treated with dox (arrows, upper middle and upper right panels), whereas the blood vessels were relatively undamaged in the mice treated with the dox-RGD-4C conjugate. The changes were scored blindly by a pathologist; representative micrographs are shown. Scale bar, 7.5 μm. **(F)** Mice bearing large (~5 cm^3) MDA-MB-435 breast carcinomas (four animals per group) were randomized to receive a single dose of free dox or dox-RGD-4C conjugate at 200 μg of dox-equivalent per mouse. A Kaplan-Meier survival curve is shown.



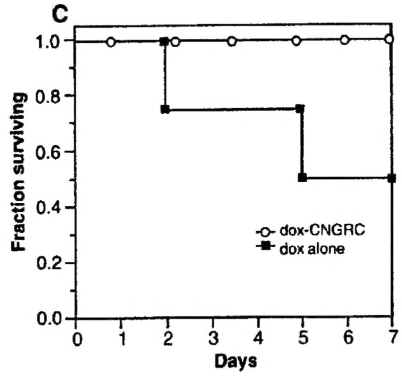


Fig. 4. Treatment of mice bearing MDA-MB-435–derived breast carcinomas with dox-CNGRC peptide conjugate. Mice with size-matched tumors (~1 cm^3) were randomized into four treatment groups (six animals per group): vehicle only, free dox, dox-ctrl pep, and dox-CNGRC. **(A)** Mice were treated with 5 μg/week of dox-equivalent. Differences in tumor volumes between day 1 and day 28 are shown. **(B)** A Kaplan-Meier survival curve of the mice in (A). **(C)** Mice bearing large (~5 cm^3) MDA-MB-435 breast carcinomas (four animals per group) were randomized to receive a single dose of free dox or dox-CNGRC conjugate at 200 μg of dox-equivalent per mouse. A Kaplan-Meier survival curve is shown.

was no different from dox alone. The dox-CNGRC conjugates were also effective against xenografts derived from another human breast carcinoma cell line, MDA-MB-231 (*12*).

We expect the NGR and RGD-4C motifs to target human vasculature as well, because (i) the NGR phage binds to blood vessels of human tumors and less so than to vessels in normal tissue (*22*), and (ii) the RGD-4C peptide binds to human α_v integrins (*9, 10*), which are known to be selectively expressed in human tumor blood vessels (*23*). Thus, these peptides are potentially suitable for tumor targeting in patients. The RGD-4C peptide is likely to carry dox into the tumor vasculature and also to the tumor cells themselves, because the MDA-MB-435 breast carcinoma expresses α_v integrins (*10*). Because many human tumors express the α_v integrins (*23*), our animal model is a reasonable mimic of the situation in at least a subgroup of cancer patients. The targeting of drugs into tumors is a new use of the selective expression of α_v integrins and other receptors in tumor vasculature. The effectiveness of the CNGRC conjugate may be derived entirely from vascular targeting because the NGR peptides do not bind to the MDA-MD-435 cells (*12*).

The tumor vasculature is a particularly suitable target for cancer therapy because it is composed of nonmalignant endothelial cells that are genetically stable and therefore unlikely to mutate into drug-resistant variants (*24*). In addition, these cells are more accessible to drugs and have an intrinsic amplification mechanism; it has been estimated that elimination of a single endothelial cell can inhibit the growth of 100 tumor cells (*24*). New targeting strategies, including the ones described here, have the potential to markedly improve cancer treatment.

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Peptide-Guided Cancer Drugs Show Promise in Mice

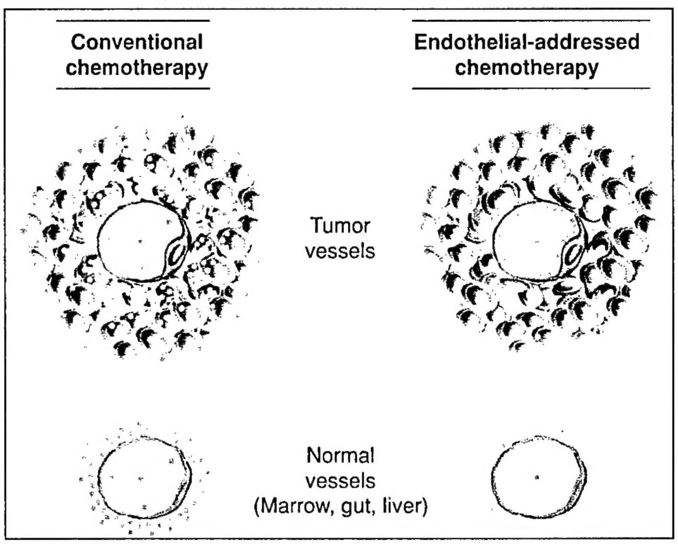
As any cancer patient who has endured chemotherapy knows, most regimens walk a fine line between killing the tumor and killing the patient. That's because chemotherapeutic drugs spread throughout the body, reaching not only the tumor but also healthy organs such as the gut and bone marrow, where they kill off normal dividing cells. To make matters even worse, tumor cells are also quick to mutate and become resistant to the drugs. Now, on page 377, a team led by Erkki Ruoslahti at The Burnham Institute in La Jolla, California, reveals a strategy that may get around both problems.

Ruoslahti and his colleagues have devised a way to target cancer drugs to the new blood vessels that nourish the tumor. They found small peptides that zero in on the cells lining these newly formed blood vessels, then linked the peptides to the chemotherapeutic drug doxorubicin. By addressing the toxic drug specifically to the tumor, the strategy spares other tissues. And because the tumor vessel cells are not cancerous themselves, they are much less likely to develop resistance to the drugs than are the highly mutable cancer cells. Indeed, when the researchers gave the peptide-drug combination to mice with large tumors, it killed off the blood vessels, stopped tumor growth, and allowed the mice to survive the cancer.

Other researchers, such as Judah Folkman at Harvard Medical School, have shown that inhibiting angiogenesis, as new blood-vessel growth is called, can block tumor growth in animals. Indeed, such work has made angiogenesis a hot research area in recent years (*Science*, 24 January 1997, p. 482). But progress on that approach has been slowed because the best angiogenesis inhibitors are proteins that are expensive and laborious to produce in bacteria. Ruoslahti's method lacks that disadvantage. "In theory, this is immediately translatable into the clinic," says tumor biologist Bob Kerbel, of the Sunnybrook Health Science Center at the University of Toronto, "because you are dealing with a drug that is already available and clinically approved. And it would not be difficult to produce these peptides."

What's more, the technique used by the Ruoslahti team to identify peptides that home in on tumor vessels can identify peptides that bind specifically to the blood vessels of other organs. This means that peptides could be developed to carry drugs to many different tissues to treat conditions other than cancer. "Ruoslahti can address drugs wherever he wants to in a nontoxic way," enthuses Folkman. "A few years from now, this will be the basis of a new pharmacology."

The idea of ferrying chemotherapeutic drugs to tumors on the backs of other molecules isn't new. But large, ungainly proteins such as antibodies have generally been used as the vehicles, with mixed success. To find small peptides that could deliver drugs specifically to tumors, Ruoslahti and then-postdoc Renata Pasqualini turned to a technique developed in the late 1980s by George Smith at the University of Missouri in Columbia. Called phage display, it involves engineering bacterial viruses called phages so that each displays a different random peptide on its surface. The technique had been used to find peptides that stick to particular proteins, by exposing a mix of peptide-displaying phages to a surface coated with the protein. Ruoslahti reasoned that if the phages were injected into an animal, the technique might be able to identify peptides that stick to specific tissues.



Zeroing in. In standard chemotherapy (left), drugs (orange) flood both tumors and normal tissues. Drugs targeted to tumor blood vessels (right) spare normal tissues.

ADVANCED MEDICAL GRAPHICS

Pasqualini made the idea work. Two years ago, she and Ruoslahti reported in *Nature* that they had identified peptides that home in on the blood vessels of the kidneys or brains of mice. "We practiced first with normal organs," says Ruoslahti, "although, all along, our aim was to look at tumors." And in this issue, the team reports that when they tried the scheme on mice with tumors, they found several peptides that stick to molecules found only in tumor-associated blood vessels.

One of the peptides the team identified binds to $\alpha_v\beta_3$ integrin, a cell-adhesion protein that David Cheresh's group at The Scripps Research Institute in La Jolla had already shown to be concentrated in angiogenic blood vessels. But the group also identified "a whole panel of other peptides," Ruoslahti says, that bind to as-yet-unidentified molecules specific to angiogenic vessels. Not only do those peptides represent alternate means of conveying drugs, but they also will be useful probes for studying the proteins they target.

Next, the team chose two of the peptides and hooked them individually to the anticancer drug doxorubicin, to see if they would guide the drug to tumors and kill them. They did. When given to mice that had large tumors derived from human breast cancer cells, even tiny amounts of the peptide-linked drug were better at stunting tumor growth than was free doxorubicin, which was hampered by its toxicity.

Indeed, some of the mice treated with the doxorubicin-peptide conjugate lived for 6 months after the treatment, while those treated with doxorubicin alone died either of tumors or of drug poisoning at the high doses. "We were never able to find a concentration of free doxorubicin that was anywhere near as effective as our conjugate," Ruoslahti says. The tumors don't disappear completely, he notes, but what remains seems to be inactive scar tissue. "The mice live very long, so it doesn't seem to bother them."

Ruoslahti thinks that the drug conjugates act primarily by killing blood vessels that feed the tumor, although the drug may also diffuse into the tumors and kill cells directly. "The technique probably targets both the vessels and the tumor cells. That is its big advantage," says tumor-cell biologist Bruce Zetter of Harvard Medical School. "It gives you a kind of double-pronged therapeutic effect that should be quite powerful." And, of course, a similar double-punch could be achieved with other drugs. "There may be things we haven't used because of their high toxicity that could be used in a more directed way," says Zetter.

Ruoslahti's isn't the only team to kill tumors by directing drugs to their blood vessels. Early last year, Philip Thorpe's group at the University of Texas Southwestern Medical Center in Dallas reported that a blood-clotting factor targeted to tumor blood ves-

sels with an antibody caused massive clotting and killed the tumors. And in November, a University of Minnesota team led by S. Ramakrishnan reported in the *International Journal of Cancer* that it had slowed tumor growth in mice by targeting diphtheria toxin to tumor blood vessels using vascular endothelial growth factor, a protein that binds to a receptor that is plentiful in new vessels.

Thorpe notes that his work relied on experimentally engineered tumor cells, so it "didn't directly extrapolate to humans" as Ruoslahti's does. And many in the field favor the latter approach over others that use proteins, because the small peptides are easy to make and use and the technique can be tailored to many different tissues.

The path to clinical trials of the peptide-doxorubicin conjugate looks fairly clear, but oddsmakers know only too well that the favorite out of the gate will not necessarily be the first across the finish line. "We are in very early stages of anti-angiogenesis therapy," says Zetter. "We have to test them all, and go after the best." Where peptide conjugates finish will be apparent only after the race is run.

–**Marcia Barinaga**